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Published in:
Biologia Plantarum

DOI:
[10.32615/bp.2020.108](https://doi.org/10.32615/bp.2020.108)

Publication date:
2020

Citation for published version (APA):
Humphreys, M. W., & Zwierzykowski, Z. (2020). Festulolium, a century of research and breeding and its increased relevance in meeting the requirements for multifunctional grassland agriculture. *Biologia Plantarum*, 64, 578-590. <https://doi.org/10.32615/bp.2020.108>

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REVIEW

***Festulolium*, a century of research and breeding and its increased relevance in meeting the requirements for multifunctional grassland agriculture**

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Abstract

Festulolium are grasses formed through interspecific hybridisation of ryegrass (*Lolium*) and fescue (*Festuca*) species. The *Lolium-Festuca* genome complex represents a vast array of heterogeneous and largely outbreeding grass species that have evolved, diverged, and adapted, allowing their world-wide colonisation of temperate grasslands. While strategies for grass improvement have focused primarily on intraspecific breeding and, in particular, on the agronomically desirable species *Lolium perenne* and *Lolium multiflorum*, a growing interest has emerged in interspecific hybrids as alternatives. The principal driver has been the increased appreciation of the capability of wide hybridisation to extend phenotypic variation beyond the ranges available within a single species. *Lolium* and *Festuca* species share complementary and desirable traits, and the prime aim in *Festulolium* (*Festuca* × *Lolium*) cultivar development has been to combine the agronomic performance of *Lolium* and the stress resistance of *Festuca* species. Advances in *Festulolium* development are timely, and support strategies aimed at delivering a more sustainable future for livestock agriculture, with grass cultivars that are persistent and productive. *Festulolium* hybrids occur naturally, including examples that demonstrate extreme heterosis with adaptations sufficient to sustain growth in harsh conditions. However, they are largely sterile and their perpetuity depends mainly on vegetative propagation. Synthetic *Festulolium* hybrids suitable for plant breeding require genome stability and fertility, sufficient for a cost-effective seed production. To this end, suitable amphiploid and introgression-breeding approaches have been developed. Herein, we provide detailed selected highlights in the research and breeding of *Festulolium*. In addition, recognising the multifunctional properties of grasslands and the development of enabling technologies that permit their study, we review additional benefits likely to accrue from *Festulolium* that may mitigate climate change effects and provide valuable ecosystem services.

Additional key words: amphiploidy, genome stability, introgression, resistance to harsh conditions.

Introduction

In an agricultural context, the species deemed most suitable for use as livestock fodder reside within the *Lolium-Festuca* complex and are: Italian ryegrass (*L. multiflorum* Lam., $2n = 2x = 14$), perennial ryegrass (*Lolium perenne* L., $2n = 2x = 14$), meadow fescue (*F. pratensis* Huds., $2n = 2x = 14$), and tall fescue (*F. arundinacea* Schreb., $2n = 6x = 42$). These *Lolium* and *Festuca* species offer a range of complementary agronomic traits, such as high nutritive value of ryegrasses, and persistency and stress tolerance commonly associated with the fescues (Thomas and Humphreys 1991). These attributes can be combined in interspecific hybrids and genotypes with

greatest potential advanced through breeding programmes with novel cultivars later assessed for their suitability for inclusion in agricultural systems.

The *Festuca* genus contains around 600 species (Cheng *et al.* 2016) and is the largest within the *Poaceae* family and comprise a series from diploid ($2n = 2x = 14$) to dodecaploid ($2n = 12x = 84$) (Clayton and Renvoize 1986, Loureiro *et al.* 2007). Species within the *Festuca* genus are widely distributed and combine a diverse range of edaphic and climatic adaptations. The *Lolium* genus comprises, in contrast to *Festuca*, only ten species, and all are diploid ($2n = 2x = 14$) (Jauhar 1993, Cheng *et al.* 2015).

Intergeneric hybrids between species of the *Lolium* and *Festuca* genera are termed *Festulolium* and include hybrids

Submitted 3 May 2020, last revision 13 July 2020, accepted 14 July 2020.

Abbreviations: GISH - genomic *in situ* hybridisation; GM -genetic modification; QTL - quantitative trait locus.

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that may have evolved naturally, but also increasing numbers as synthetic hybrid cultivars including some with novel genome combinations, all generated through the use of conventional plant breeding technologies.

Prior to 2004, there were strict limitations to the widespread marketing and commercial use of *Festulolium* cultivars within the European agricultural context. This was a consequence of certain European Union directives (66/401/EEC and 92/19/EEC), whereby marketing of grass cultivars under the *Festulolium* label was restricted to just a single *Lolium* and *Festuca* species combination. This hybrid, also found naturally, and classified as *Festulolium braunii* (K. Richter) A. Camus, derives from cross-pollination of *Lolium multiflorum* Lam. (Italian ryegrass), a highly productive biennial forage grass, and *Festuca pratensis* Huds. (meadow fescue), its close relative, a more resilient perennial species, but inferior to the ryegrass in its forage quality.

Subsequent to 2004, and in recognition that previously EU regulations had prevented commercial exploitation of alternative and promising *Lolium* spp. × *Festuca* spp. hybrids, a more inclusive definition was agreed. This amended EU directive (2004/55/EC) enabled all *Lolium*/*Festuca* species combinations with potential value for use in agriculture to be developed and marketed as *Festulolium*. The changes arose following research outcomes undertaken as part of an EU FPV Programme funded as *SAGES* (QLRT 30764). The project included use of a range of alternative species' combinations with traits deemed suitable for crop production tailored to contrasting European locations and growth conditions. *SAGES* research was undertaken by a consortium of leading European-based *Festulolium* researchers, supported by commercial partners based in the UK, Poland, France, and Norway, and its objectives endorsed by additional allied EU grass seed companies. The redrafted definition for *Festulolium* embraces in a much more effective manner, the full range of opportunities presented to plant breeders following access to the vast array of genetic and phenotypic variation present within the *Lolium*-*Festuca* genome complex.

The recognition that *Lolium* and *Festuca* species hybridise naturally has allowed commercial exploitation of *Festulolium* to proceed in Europe without the constraints applied to other crops that rely on genetic modification (GM) technologies to introduce or enhance expression by a chosen trait.

For this review, some key highlights are provided in *Festulolium* research and breeding. However, while in part retrospective, the review aims to highlight some recent novel findings and scopes suggestions for future targets for *Festulolium* research and breeding. These can be achieved through incorporation of the latest high-throughput genomic-assisted breeding (e.g., Kole *et al.* 2015), combined with phenomic technologies to screen target traits (e.g., for improved root architecture and ontogeny; Marshall *et al.* 2016). Through a targeted “designer-breeding” approach, specific *Lolium*/*Festuca* species combinations can be created, aimed at specific traits deemed appropriate for “climate-smart” strategies relevant to location for intended use and to specific function

requirements. For example, the provision of sustainable yields of high-quality fodder will depend on whether the crop has the necessary resilience sufficient to withstand an abiotic stress it is likely to encounter (Humphreys *et al.* 2012, Macleod *et al.* 2013). Breeding targets should become more holistic in order to encompass whole ecosystems, and in so doing, agricultural systems can then be developed that provide efficient win/win scenarios (Humphreys *et al.* 2014a). For example, it is entirely feasible to provide sustainable, healthy, and efficient fodder for livestock, while alongside also mitigating some of the worst scenarios of climate change (e.g., combating incidents of flooding; Macleod *et al.* 2013). While many of the ecosystem services likely to benefit from cultivation of *Festulolium* relate to their root/soil interactions, others emanate from their foliar properties and their interactions as feed for livestock. There is growing evidence that certain novel *Festulolium* hybrids are capable of delivering better efficiencies in protein use by ruminants than currently provided by existing grass cultivars (e.g., Humphreys *et al.* 2014b, Kamau *et al.* 2020).

Development of forage grass breeding using *Lolium*, *Festuca*, and *Festulolium*

The concept that genotypes selected from heterogeneous and primarily obligate outbreeding species should be selected on the basis of their phenotypes, and those having advantageous trait expression be inter-crossed and advanced through plant breeding programmes in cultivar development, dates back a hundred years. The plant breeding approach may have lacked finesse, and has sometimes been described as “crossing the best with the best, and hoping for the best”, but nevertheless, it has been largely successful.

With a few exceptions e.g., *L. temulentum*, and *F. microstachys*, that as inbreeding species, have largely entered an evolutionary *cul de sac*, the majority of species within the *Lolium*/*Festuca* complex are outcrossing, in unison providing a vast resource of diverse allelic and phenotypic variants. These variants collectively convey alternative edaphic and climatic adaptations which, by incorporating the latest screening technologies, can be selected and advanced in breeding programmes. New high-throughput genotype and phenotype selection technologies provide greater reliability in trait selection across generations. Their use often circumvents costly, lengthy, and frequently unreliable field trials during cultivar development.

Over the years of forage grass breeding, there has been progression in performance, with successive cultivars demonstrating ever-increasing agronomic gain, providing largely cost-effective, healthy, and sustainable livestock fodder. Grassland agriculture frequently does not compete to any significant extent with standard cropping systems as grass cultivars are particularly well adapted to growth on marginal land, areas deemed unsuitable for alternative crop production, such as cereals. In such marginal areas, grasslands provide a vital contribution in the support of

frequently fragile rural economies. The ryegrasses, and in particular perennial ryegrass (*L. perenne*) and Italian ryegrass (*L. multiflorum*), considered optimal providers of forage of high nutritive value for livestock, are unfortunately susceptible to severe winter and/or summer stresses. Where abiotic stresses are encountered regularly, more resilient grass species are often used including timothy (*Phleum pratense* L.) and cocksfoot (*Dactylis glomerata* L.). Fescue species may also be used, in particular the broad-leaved fescues such as meadow fescue (*F. pratensis* Huds.), and tall fescue (*F. arundinacea* Schreb.), the close relatives of ryegrass (Humphreys *et al.* 2006b).

The Welsh Plant Breeding Station (WPBS), which in 2019 celebrated its centenary was at the forefront in the development of grass and clover cultivars, with the first perennial ryegrass cv. S23 released in 1931 (Humphreys *et al.* 2006b). For many subsequent years, equivalent breeding approaches were practised widely across Europe, the Americas, Asia, and Australasia, involving species and hybrids with adaptations tailored to their end-user and location. In general, plant breeding procedures were based on recurrent selection of individual plants or their half-sib families (Humphreys *et al.* 2006b). Initial advances were accelerated significantly in the UK following the end of the World War II, when the need for food self-sufficiency was recognised as a major driver and was considered essential to future national security (Stapledon and Davies 1948).

As with ryegrass, *Festulolium* research dates back at least 100 years. Even as long ago as 1790, suggestions were made for possible progenitors for the naturally occurring *Festulolium* species, *Festulolium loliaceum* (Hudson) P. Fournier, (Jenkin 1924, 1933). Clearly, from the infancy of grass breeding, researchers recognised the potential value of interspecific *Lolium/Festuca* hybrids. This view has steadily gained strength, and now is widely accepted (Essad 1964, Thomas and Humphreys 1991). The benefits are particularly evident in situations where it is deemed necessary to extend the range of genetic diversity and plant adaptation beyond that available within a single grass species.

Heterosis has long been recognised as advantageous, and is frequently demonstrated amongst *Festulolium* hybrids, where it is evident by the plant vigour, competitive capabilities, and adaptations to stresses. In permanent grasslands, triploid and diploid cytotypes of *Festulolium loliaceum* (*L. perenne* × *F. pratensis*) grow well on waterlogged soils where the performance of the parent species was often suboptimal (Gymer and Whittington 1973, Humphreys and Harper 2008). Extreme examples of heterosis have been observed at high altitudes in Alpine meadows where naturally occurring triploid hybrids between *F. pratensis* (2x) and *F. pratensis* subsp. *apennina* (De Not.) Hegi (4x) demonstrate remarkable growth and vigour (Boller and Kopecký 2020). Generally, naturally occurring *Festulolium* hybrids have little or no male or female fertility (Humphreys and Harper 2008, Kopecký *et al.* 2016), and rely to a large extent on vegetative propagation. Using *F. pratensis* subsp. *apennina* as an example, a capability to hybridise and to form hybrids with closely-related species growing in their close proximity

provides opportunities for novel genome combinations and plants that frequently thrive in harsh mountain habitats where their complementary or additive traits provide them a selective advantage. Over the millennia, events involving *Lolium* and *Festuca* species that have generated advantageous genome combinations through species' hybridisation, and provided them a selective advantage, have become fixed, and may further led to speciation. Whilst the *Lolium* genus comprises only diploid species, the *Festuca* genus is far larger and more complex, and includes many species that have evolved following interspecific hybridisation. For such species to evolve from interspecific hybrids, it is necessary that they acquire fertility. This was achieved by chromosome doubling, and led to the creation of a polyploid series in the *Festuca* genus. Chromosome doubling in fescue species is thought to have arisen largely as a consequence of unreduced gametes formed by their constituent parent species, during meiosis (Kopecký *et al.* 2016). Chromosome doubling within hybrids not only provided fertility, but also enabled advantageous genome complements having been assembled during hybridisation to be retained over generations, in perpetuity. This is achieved through sets of homologous chromosomes in fescue hybrid species pairing at meiosis in a preferential manner as intraspecific homologous chromosome associations. These segregate during gamete formation so that all constituent genome complements are maintained and transferred into subsequent generations.

Amongst polyploid fescues, the most studied is tall fescue, *F. arundinacea* (6x). A detailed cytological study involving genomic *in situ* hybridisation (GISH) (Humphreys *et al.* 1995) showed that it evolved following hybridisation of *F. pratensis* (2x), endemic to northern and western Europe, and *F. arundinacea* var. *glaucescens* (4x), a grass species indigenous to southern Europe with adaptations to Mediterranean climates. The hybridisation of the two fescue species, and subsequent intermediary events that enabled genome duplication, led over the millennia to the speciation of hexaploid *F. arundinacea* Schreb. (tall fescue). Genome interactions between the constituent genomes of tall fescue provided it with considerable adaptive plasticity and tolerance to contrasting temperature extremes and water availabilities.

Detailed genetic studies demonstrated that the strict homologous intraspecific chromosome pairing practised by tall fescue led to diploid-like meiosis and disomic inheritance (Lewis *et al.* 1980) ensuring the advantageous species' combinations were always maintained. Jauhar (1975) proposed that in addition to structural affinities that encouraged preferential homologous chromosome pairing in tall fescue and related polyploid fescue species, this property was supported further through the activity of a genetic chromosome pairing control regulator. Jauhar (1975) proposed the regulator in the fescues to be hemizygous ineffective, so that subsequent to hybridisation with another species it becomes non-functional, which would then permit homoeologous chromosome recombination to proceed unhindered and enable interspecific genetic transfers.

The broad-leaved fescues regularly employed in

Festulolium research and breeding (Ghesquière *et al.* 2010a, b) belong to *Festuca* subg. *Schedonorus* and comprise two geographical lineages, the African clade (*F. mairei*, *F. arundinacea* subsp. *atlantigena*, and *F. arundinacea* var. *letourneuxiana*), and the European clade (*F. pratensis*, *F. arundinacea*, *F. pratensis* var. *apennina* and *F. arundinacea* var. *glaucescens*) (Torrecilla and Catalan 2002). An unknown diploid progenitor species is believed to be the source of the chromosome pairing regulator and to be common and central to speciation events within the European and most likely the African *Schedonorus* clade. It is speculated that this species contributed a common genome to *F. pratensis* var. *apennina*, *F. arundinacea* var. *glaucescens* and *F. arundinacea* (Kopecký *et al.* 2016).

All species from the European clade, with the exception of *F. pratensis* var. *apennina*, have been involved in the *Festulolium* cultivar development, but from the African clade, so far only *F. mairei* has been so involved. Breeding successes using *F. mairei* were reported initially by Chen and Sleeper (1999), and later by Humphreys *et al.* (2014b, 2018) who developed cv. AberRoot, which recently gained entry onto the 2020 UK National List.

Humphreys (1989) was the first to describe the use of a pentaploid *L. multiflorum* × *F. arundinacea* ($2n = 5x = 35$) hybrid as part of a backcross-breeding programme aimed at transferring *F. arundinacea* traits into *Lolium*. Humphreys and Ghesquière (1993) later demonstrated the efficacy of the procedure using a polyallelic isozyme marker capable of discriminating isoalleles at orthologous chromosome sites on each of the five constituent genomes in the pentaploid hybrid. They demonstrated different frequencies in recombination rates between the *Lolium* and *Festuca* homoeologous chromosomes carrying the marker. Introgression of alleles from tall fescue into ryegrass favoured chromosome transfers from the *F. pratensis* genome more than from those of *F. arundinacea* var. *glaucescens* (a more distant relative). The research was the first to demonstrate conclusively that all three tall fescue genomes were accessible for gene transfers into ryegrass, and that a pentaploid hybrid between *L. multiflorum* and *F. arundinacea* was an effective starting point for introgression breeding programmes involving these species.

It has been long recognised that knowledge of the taxonomical and evolutionary relationships among species within the *Lolium-Festuca* complex would aid species selection and guide the strategy for interspecific plant breeding programmes. The complex phylogenetic relationships of *Lolium* and *Festuca* species and their grass relatives have been studied repeatedly by many researchers. Species' hybrids were studied, initially using morphological traits and also their cytological behaviour (e.g., Malik and Thomas 1966, Borrill *et al.* 1980). The cytology was later given significant support through the use of genomic *in situ* hybridisation (GISH) technologies (e.g. Thomas *et al.* 1994, Zwierzykowski *et al.* 1998, 2008, Canter *et al.* 1999, Kopecký *et al.* 2005). The application of GISH to mitotic and meiotic chromosome spreads of interspecific hybrids contributed new understanding of genome constitutions (e.g., Humphreys *et al.* 1995),

and opportunities to physically locate the DNA of large chromosome translocations (>1 kb). By integrating cytogenetic and phenotypic studies, researchers were able to locate several quantitative trait loci (QTL) that governed expression of important traits, such as drought tolerance on chromosome 3 (Humphreys and Pašakinskienė 1996).

More detailed understanding of phylogenetic relations within the *Lolium-Festuca* complex was made possible by the applications of chloroplast (e.g., Cheng *et al.* 2016), nuclear DNA markers (e.g. Stammers *et al.* 1995), rDNA nucleotide sequence analysis (e.g., Torrecilla and Catalan 2002), and markers such as sequence related amplified polymorphism (SRAP) (Cheng *et al.* 2015). More broadly, these studies provided understanding of relationships with other grass and cereal crop species within the *Poaceae*, and permitted comparative studies of genome organisation and function (e.g., Yamada *et al.* 2005, Armstead *et al.* 2007).

Species classification and the nomenclature used within this review have been employed consistently over the last century and appear regularly in publications by most European researchers, including those by the current authors, and are also used widely elsewhere (e.g. in Cheng *et al.* 2016). The term *Festulolium* conforms to specific EU directives and is applied to all ryegrass/fescue hybrid cultivars, irrespective of their species derivation, and is used in their commercial marketing. As a consequence, it would seem unlikely, that a change to the current nomenclature, will be forthcoming in Europe. However, it should be stated that the above species' nomenclature, classification and use are not consistently applied worldwide. In particular, in the USA, any reference to *Lolium* and *Festuca* species are usually replaced by alternative nomenclature and incorporated regularly on certain databases. It was suggested that the broad-leaved fescues in subgen. *Schedonorus* should better be included within *Lolium* (Darbyshire and Warwick 1992). The suggestion arose despite the obvious differences in the inflorescence morphology between ryegrasses and fescues, the fescues having open panicles and the ryegrasses with compact spikes. Other taxonomists have suggested a split of the subgen. *Schedonorus* into an independent genus, *Schedonorus* (e.g. Sorreng and Terrell 1997). In order to assist in their cross-reference, those fescue species used most frequently in *Festulolium* breeding (see Table 1 Suppl.) are listed below with some (but not all) of their alternative nomenclature. Meadow fescue (*Festuca pratensis*) is *Lolium pratense* (Huds.) Darbysh., or *Schedonorus pratensis* (Huds.) P. Beauv. Tall fescue (*F. arundinacea*) is *Lolium arundinaceum* (Huds.) Darbysh., or *Schedonorus arundinaceus* (Schreb.) Dumort.

A growing understanding of genome synteny in *Poaceae* crop species (e.g., Yamada *et al.* 2005, Armstead *et al.* 2006) allowed researchers to “fast-track” knowledge transfers of genome structure from extensively studied crop species, to those far less explored species, including species within the *Lolium-Festuca* complex. The strategy enabled the successful prediction of QTL location relevant to a range of complex traits, and enabled their respective genome sequences to be compared effectively. For example, Alm *et al.* (2011) described two major

frost/tolerance/winter survival QTL on *F. pratensis* chromosome 5 that are likely to correspond to equivalent *Fr-A1* and *Fr-A2* loci found on chromosome 5A of wheat. They also described a large QTL for drought tolerance on *F. pratensis* chromosome 3 that would likely incorporate orthologous gene sequences reported in *F. arundinacea* and *F. arundinacea* var. *glaucescens*. These had, subsequent to their introgression onto chromosome 3 of *L. multiflorum* from the fescue species, been shown conclusively to confer to it an enhanced drought tolerance (Humphreys and Pašakinskienė 1996, Humphreys *et al.* 2005).

Identifying QTL locations by associations to gene markers aligned along specific chromosome linkage groups has been a helpful support to *Lolium-Festuca* spp. genome studies. QTL-maps were constructed for traits of importance both in *Festuca pratensis* (Alm *et al.* 2011), and in *Lolium perenne* (Turner *et al.* 2008). Unfortunately, there are frequently practical difficulties in translating knowledge of QTL location derived from experimental genetic mapping populations for use in alternative germplasm, including breeders' lines used in cultivar development.

Introgression-mapping is a technology that attempts to integrate genomic and phenotypic studies. From a plant breeding perspective, it has advantages over QTL analysis in that alien genome sequences are targeted specifically for selection across generations. This can be achieved whilst otherwise maintaining the host genome intact and limiting incidents of linkage drag that might otherwise deteriorate crop performance. Two exemplar studies demonstrate the efficacy of introgression-mapping and its direct applications into plant breeding and cultivar development. The first involved a simple gene trait, the recessive stay-green mutant *Sid*, and the second, a complex quantitative trait for drought tolerance. In the first example, introgression-mapping enabled the location and transfer of a stay-green mutant *Sid* onto a distal location of chromosome 5 in *Lolium*. In order to function, recessive gene *Sid* has to be transferred onto homologous *Lolium* spp chromosome sites. A *L. perenne* turf grass cv. AberNile, homozygous for the *Sid* gene, was commercialised and used widely as an amenity and sports grass in the 1980s. To this day, the stay-green gene remains the most extensively researched example of the use of introgression-mapping relevant to the *Lolium-Festuca* complex. It is an excellent example of the use of marker-assisted selection as a technique to aide trait selection within cultivar development (Thomas *et al.* 1997). The *Sid* gene, which has been isolated and sequenced, was later discovered to be orthologous to the gene for yellow/green cotyledon polymorphism reported by Gregor Mendel in 1886 (Armstead *et al.* 2007). Introgression-mapping for complex traits, and QTL selection, has been more challenging, but there have been notable successes. One involved plants derived from a backcross introgression-breeding population exposed to simulated prolonged drought conditions. In the best drought survivors, it was found, firstly by GISH, and subsequently using associated genetic markers that an identical *Festuca*-derived DNA sequence was recovered. Consistent amongst the best drought survivors were

genotypes that carried a translocated *F. arundinacea* var. *glaucescens* DNA sequence at the same distal location of the *L. multiflorum* chromosome 3 (Humphreys *et al.* 2005). The fescue-derived sequence retained effective expression of drought tolerance over generations and in a broad array of alternative genetic backgrounds. The marker-assisted transfer of the fescue genes into a *L. multiflorum* breeding population led to the development of the *IBERS*-bred and UK National Listed *Festulolium* cultivar AberLink. The fescue-derived sequence was transferred subsequently from the *L. multiflorum* cultivar into a *L. perenne* breeding population where its drought tolerance was retained (Humphreys *et al.* 2012).

However, introgression-mapping for major QTL in *Lolium-Festuca* populations has not always been as successful. Harper *et al.* (2018) found, when investigating genotype-phenotype association within a genome-characterised *L. perenne*/*F. pratensis* introgression-mapping population, that phenotype expression attributed to a fescue chromosome was on occasions subsequently lost in progeny, despite the retention of the relevant fescue-based translocations.

Introgression-mapping frequently supports plant physiology investigations. Through its targeted approach, it provides an opportunity for complex traits to be effectively “dissected” so that their underlying mechanisms are revealed. A good example was a drought tolerant experimental *Festulolium* cultivar Bx509 (Humphreys *et al.* 2018). This cultivar contained a *F. arundinacea*-derived DNA sequence introgressed onto the long arm of chromosome 3 of *L. perenne* cv. AberStar, at a proximal location. Using high-throughput and detailed phenotyping, it was demonstrated that Bx509 had a significantly larger and deeper root system compared to cv. AberStar, which in all likelihood aided its drought tolerance. The introgression-mapping technologies also helped to identify genes involved in the photosystem II cold-acclimation in *F. pratensis* (Humphreys *et al.* 2006a), and in *Festulolium* cultivars (Rapacz *et al.* 2004).

Whilst the potential for *Festulolium* use has been recognised widely, until recently it is only in Central and Eastern Europe that there has been a consistent strategy for *Festulolium* breeding, and there is a continuous pipeline of developing new cultivars. However, recent political, economic and environmental drivers have refocused thoughts and priorities elsewhere, and, especially the UK, aimed at seeking new and more environment-friendly and sustainable solutions in agricultural production. In this new scenario, and taking into account essential requirements to mitigate climate change, there are ever-increasing opportunities for *Festulolium* cultivar use (Humphreys *et al.* 2014b), which should broaden their global appeal.

Breeding of *Festulolium* cultivars

Throughout the history of *Festulolium* breeding, the primary objective has been to combine the high yield and quality of ryegrasses with abiotic stress tolerance of fescues. This was to enhance grassland persistence, and to

maintain sustainable forage production (Ghesquière *et al.* 2010a). As previously described, *Festulolium* cultivars can be developed *via* the amphiploid approach, with the intention to combine entire *Lolium* and *Festua* genome complements, or *via* the introgression-breeding approach, where specific genes from a donor species are incorporated into the otherwise undisturbed genome of the host species (Ghesquière *et al.* 2010a).

The first amphiploid *Festulolium* cultivar Elmet [= × *Festulolium braunii* (K. Richter) A. Camus], is amphidiploid ($2n = 4x = 28$) and was developed in the early 1970s at the Welsh Plant Breeding Station, Aberystwyth, UK (Lewis *et al.* 1973). The cultivar was obtained by hybridising colchicine-induced autotetraploid ($2n = 4x = 28$) forms of diploid *L. multiflorum* ($2n = 2x = 14$) and *F. pratensis* ($2n = 2x = 14$). The same research group, using the same breeding approach, produced the allotetraploid cv. Prior from hybrids of *L. perenne* ($4x$) × *F. pratensis* ($4x$) [= × *Festulolium loliaceum* (Huds.) P. Fourn.]. Cv. Prior gained entry onto the Swedish National Recommended List and was also employed in breeding programmes elsewhere, such as in the USA (Casler *et al.* 2001), and in Norway (Østrem *et al.* 2013). The cultivar was later assessed in the UK for additional ecosystem service properties (Macleod *et al.* 2013). However, neither cv. Elmet nor cv. Prior were ever registered onto the UK National Recommended Lists, a prerequisite to their commercial use. In the 1980–2000s, multiple successes in breeding of *Festulolium* amphiploid cultivars followed, especially in Central and Eastern Europe, and several cultivars were registered as × *Festulolium braunii*. In Czechoslovakia (now, the Czech Republic), at the Hladké Žitovice Breeding Station (recently *DLF Seeds*, s.r.o.), Antonín Fojtík and his co-workers developed several *Festulolium* cultivars which were commercialized in many countries, and still remain on the OECD list.

The restrictive definition of *Festulolium* prior to 2004, which limited marketing to single species combination (*Festulolium braunii*), was a significant obstacle to *Festulolium* breeding. *F. arundinacea* (tall fescue) has over many years been considered as incorporating many traits deemed desirable for inclusion in a persistent grass cultivar (e.g. drought tolerance, large root biomass). Effective breeding programmes to introduce *F. arundinacea* traits into *Lolium* were initiated (e.g. Humphreys and Ghesquière, 1993). However, the constraints prior to 2004 prevented their inclusion as *Festulolium*. As a consequence, promising cultivars derived from *L. multiflorum*/*F. arundinacea* hybrids, were included on tall fescue National Lists. In the USA, at the Kentucky Agricultural Experiment Station, Lexington, Buckner *et al.* (1977, 1983) developed two hexaploid tall fescue cultivars, Kenhy (1977) and Johnstone (1983) through an introgression-breeding approach. These derived from fertile colchicine-induced amphiploids ($2n = 8x = 56$), produced from initially sterile tetraploid hybrids of *L. multiflorum* ($2x$) × *F. arundinacea* ($6x$).

In the Czech Republic, at the Breeding Station Hladké Žitovice, Fojtík (1994) developed an extensive breeding programme in which partly female fertile F_1 hybrids of

L. multiflorum ($2x$) × *F. arundinacea* ($6x$) were backcrossed either onto hexaploid *F. arundinacea* or tetraploid *L. multiflorum*. In the former case, fourteen hexaploid tall fescue introgression cultivars including Felina (1989), Hykor (1991) and Korina (1997) were produced, and in the latter, five tetraploid Italian ryegrass introgression cultivars, such as Bečva (1989) and Lofa (1997).

Seventy-eight *Festulolium* cultivars have been released globally (listed in Table 1 Suppl.). These comprise 33 cultivars derived through the amphiploid approach, and 45 cultivars derived from the introgression approach. Of these, 53 are registered on the 2020 OECD List, a prerequisite for seed marketing in most countries.

Ghesquière *et al.* (2010a) reviewed progress in *Festulolium* cultivar development between the 1970s and 2010. They listed a total of 42 cultivars (incorporated into Table 1 Suppl.), of which 23 were amphiploid and 19 were introgressive forms. During the last 10 years, *Festulolium* breeding has advanced considerably. Most striking are the increased numbers of cultivars derived through the introgression-breeding approach. Compared to earlier times, the number of such cultivars currently available has more than doubled, from 19 in 2010 to 46 in 2020. Some of the cultivars listed in Table 1 Suppl. no longer remain on the OECD List (e.g., Elmet, Rakopan, Prior, Lueur, Lusilium and Luxane), and new cultivars (e.g., Hopej, Honor, Hypnos, Lukida, and Naos) have yet to become registered on the OECD List.

Cultivars derived through an amphiploid-breeding approach (see Table 1 Suppl.): Thirty-three *Festulolium* cultivars, all tetraploid ($2n = 4x = 28$), have been developed using the amphiploid-breeding approach. Twenty-three cultivars derive from reciprocal *L. multiflorum* ($4x$) ♀ × *F. pratensis* ($4x$) and *F. pratensis* ($4x$) ♀ × *L. multiflorum* ($4x$) hybrid combinations. Of these, seven were developed in the Czech Republic (Fojtík 1994, V. Cernoch, personal communication), four in Poland (Zwierzykowski *et al.* 1994, 1998, Joks *et al.* 1995), four in Germany (e.g., Netzbänd 1991), three in Lithuania (Nekrošas *et al.* 1995, Nekrošas and Kemešytė 2007), two in the UK (e.g., Lewis *et al.* 1973), one in Japan (Kubota *et al.* 2015), and one each in Argentina and Italy. Among the five amphiploid cultivars derived from hybrids of *L. perenne* ($4x$) × *F. pratensis* ($4x$), two were developed in Norway (Østrem *et al.* 2013), one in the UK (Lewis *et al.* 1973), and one in Japan (Kubota *et al.* 2015).

In addition to *F. pratensis*, the other progenitor species involved in the evolution of hexaploid *F. arundinacea* is the Mediterranean fescue, *F. arundinacea* var. *glaucescens* ($4x$) (Humphreys *et al.* 1995). Although, not used as regularly as *F. pratensis* or *F. arundinacea* in plant breeding, *F. arundinacea* var. *glaucescens* is now receiving increased interest and involvement. From tetraploid hybrids of *L. multiflorum* ($4x$) × *F. arundinacea* var. *glaucescens* ($4x$), three cultivars were developed in France (Ghesquière *et al.* 1996, 2010a, b).

Although it is yet to gain entry onto National Lists, an amphiploid cultivar (Bx514) of the hybrid between *L. perenne* and *F. arundinacea* var. *glaucescens* ($4x$)

with good overall agronomic performance has also demonstrated valuable ecosystem services (Humphreys *et al.* 2014). The cultivar has demonstrated its potential to support efficient ruminant nutrition and for combating soil compaction (Humphreys *et al.* 2014, Kamau *et al.* 2020, Muhandiram *et al.* 2020).

Festulolium cv. AberAtlas has been included onto the 2020 UK National List and globally is the first *L. perenne* × *F. mairei* amphiploid cultivar (Humphreys *et al.* 2014, 2018, Kamau *et al.* 2020).

Casler *et al.* (2001), in the USA, developed cv. Spring Green by inter-pollinating four existing *Festulolium* cultivars: Elmet, Tandem and Kemal (all *L. multiflorum* × *F. pratensis*), and cv. Prior (*L. perenne* × *F. pratensis*).

Pedersen *et al.* (1990) registered germplasm KY-2N56 ($2n = 8x = 56$), which originated from two hybrids: *L. multiflorum* × *F. arundinacea* and *F. arundinacea* × *F. gigantea*.

Cultivars derived through an introgression-breeding approach (see Table 1 Suppl.): Forty-five *Festulolium* cultivars have been developed following the introgression-breeding approach. These involved backcrossing *Lolium-Festuca* hybrids into either *L. multiflorum*, or *L. perenne* (for introducing fescue traits into ryegrass), or into *F. arundinacea* (for introducing ryegrass traits into tall fescue). For several introgression-bred cultivars, e.g. Evergreen, Kemal and Tandem (*L. multiflorum* × *F. pratensis*) and Barfest, Duo, Barrier, Matrix, Revolution and Ultra (*L. perenne* × *F. pratensis*), knowledge over their fescue content is somewhat limited. The cultivars were classified as introgression lines based primarily on their phenotypes and *Lolium*-like appearance (Ghesquière *et al.* 2010a).

Twenty-four introgression-bred *Festulolium* cultivars derive from *L. multiflorum* × *F. arundinacea* hybrids. Their development centred primarily at the Breeding Station Hladké Životice. As a starting point, tetraploid hybrids between *L. multiflorum* (2x) and *F. arundinacea* (6x) were, backcrossed onto one of the parent species (e.g., Fojtik 1994, Kopecký *et al.* 2005). Amongst 21 Czech *Festulolium* cultivars, 14 (all hexaploid, $2n = 6x = 42$) derived via introgression of *L. multiflorum* traits into *F. arundinacea*, and seven cultivars (all tetraploid, $2n = 4x = 28$) via introgression of *F. arundinacea* traits into *L. multiflorum* (V. Cernoch, personal communication).

Eight cultivars developed via an introgression breeding were obtained from backcrosses of *L. multiflorum* (4x) × *F. pratensis* (4x) hybrids onto *L. multiflorum* (4x). Three of those were produced in the USA (Casler *et al.* 2001), two in Switzerland (Ch. Grieder, personal communication), and the remaining in the UK (M. W. Humphreys, personal communication), Japan (Kubota *et al.* 2015) and Uruguay.

Seven *Festulolium* cultivars were developed by introgression of *F. pratensis* genes into *L. perenne* from initial *L. perenne* × *F. pratensis* hybrids. Amongst these, four cultivars, all diploid, were developed in New Zealand (A. Stewart, personal communication). Three cultivars, all tetraploid, were obtained from backcrosses of *L. perenne* × *L. multiflorum* hybrids into

F. pratensis, two in Latvia (Bērziņš *et al.* 2018) and one in Belgium (J. Baert, personal communication).

There are two known examples of use of a targeted approach aimed at specific genetic transfers, in both cases from a donor fescue species into ryegrass that have led to cultivar registration. An integrated physical and genetic mapping approach involved using diploid drought-tolerant *L. multiflorum* genotypes incorporating *F. arundinacea* var. *glaucescens* genes as the starting point (Humphreys *et al.* 2005). These were the source of germplasm that led to the development of the drought-tolerant cv. AberLink (Humphreys *et al.* 2014b). Unique in Table 1 Suppl., as it is an amenity and not a forage grass, cv. AberNile was the first confirmed *Festulolium* introgression-line, and is a diploid *L. perenne* turf grass, homozygous for the recessive *F. pratensis*-derived allele *Sid* for the stay-green trait (Thomas *et al.* 1997).

Genomic constitution of *Festulolium* cultivars

Over the years, cultivar development of synthetic *Festulolium* amphiploids has been seriously compromised by an inability to restrict pairing to true homologous chromosome partners. Incidents of homoeologous chromosome pairing that likely lead to aneuploidy, and over generations, compromise cultivars' genetic stability and fertility are regularly witnessed (Thomas and Humphreys 1991).

Cytogenetic studies using genomic *in situ* hybridization (GISH) have confirmed the significant extent of homoeologous pairing and interspecific genome recombination that occurs within *Festulolium* progenies (e.g., Zwierzykowski *et al.* 1998, 2006, 2011, Canter *et al.* 1999, Pašakinskienė and Jones 2003, Kopecký *et al.* 2005, 2006, 2017, Kubota *et al.* 2015). Zwierzykowski *et al.* (1998) were first to describe extensive homoeologous recombination in four F_8 populations derived from *F. pratensis* × *L. multiflorum* hybrids ($2n = 4x = 28$). These populations were registered in Poland as cultivars Agula, Felopa, Sulino, and Rakopan. Genome constitutions of these cultivars deviated greatly from the expected 50 % *Lolium*: 50 % *Festuca*. The percentage of the *L. multiflorum* chromatin ranged from 49.2 to 66.7 %. This would infer increasing genomic imbalance and the favoured transmission of *Lolium* as compared to *Festuca* chromatin. These conclusions were confirmed in other independent studies. Extensive incidents of genome imbalance and homoeologous recombination were observed in the F_8 genotypes derived from the amphiploid *L. perenne* (4x) × *F. pratensis* (4x) cv. Prior (Canter *et al.* 1999). Similar findings were made in the Lithuanian cv. Punia (*F. pratensis* (4x) × *L. multiflorum* (4x) (Pašakinskienė and Jones 2003).

Zwierzykowski *et al.* (2006, 2011) reported extensive interspecific chromosome recombination between the parental genomes in successive F_2 - F_8 generations of breeding populations developed from *F. pratensis* (4x) ♀ × *L. perenne* (4x) hybrids. They also reported multiple incidents of substitution of whole *Festuca* chromosomes by

those of *Lolium*. The total number of *Lolium* chromosomes present increased from the mean of 14.4 in the F_2 to 17.6 in the F_8 , while the corresponding *Festuca* chromosome number decreased from 13.6 to 10.5.

Kopecký *et al.* (2006) analysed the genomic constitution of 26 *Festulolium* cultivars generated *via* both amphiploid and introgression-breeding approaches. They reported large variations in the proportions of parental genomes and in the extent of generic recombination in eleven amphiploid cultivars derived from reciprocal hybrids of *L. multiflorum* (4x) × *F. pratensis* (4x), and also in a *L. perenne* (4x) × *F. pratensis* (4x) cultivar, supporting earlier observations by Zwierzykowski *et al.* (1998) and Canter *et al.* (1999). In *Festulolium* cultivars developed by backcrossing *L. multiflorum* (2x) × *F. arundinacea* (6x) hybrids onto *F. arundinacea*, GISH analysis indicated a prevalence of the *Festuca* genome, and a high number of recombinant *Lolium-Festuca* chromosomes. In cultivars developed from *L. multiflorum* (2x) × *F. arundinacea* (6x) hybrids backcrossed onto *L. multiflorum* (4x), Kopecký *et al.* (2006) observed only *Lolium* chromosomes, and sporadically one or more recombined chromosomes. In three other cultivars, they were unable to detect the presence of any *Festuca* derived chromatin.

A multi-location three-year field trial was constructed in eight European countries by the members of a consortium within the EUCARPIA Fodder Crops and Amenity Grasses Section, *Festulolium* Working Group (Kopecký *et al.* 2018). Each site included the same 15 elite *Festulolium* cultivars and representative cultivars of their parental species, used as controls. In addition to comparative studies in their field performance, a detailed investigation of their genome constitution using both GISH and Diversity Arrays Technology (DArT), was undertaken. The *Festulolium* cultivars included forms from both amphiploid and introgression-breeding. Species combinations included *L. multiflorum* × *F. arundinacea* var. *glaucescens*, *L. multiflorum* × *F. arundinacea*, *L. multiflorum* × *F. pratensis*, and *L. perenne* × *F. pratensis*. Over the wide range of cultivars of distinct and different origins, the genome study found widespread predominance in the retention of *Lolium*, compared to *Festuca*-derived chromatin.

Differences in genome compositions within *Festulolium* amphiploid cultivars were often larger than those between cultivars, thereby illustrating the difficulties for breeders in achieving the necessary uniformity required for cultivar registration. The extent of the variation in genomic constitution, both within and between cultivars, is likely to be affected by the number of generations of seed multiplication undertaken subsequent to generating the initial F_1 hybrids. Unfortunately, there is only limited data available in this regard. All Czech and Polish allotetraploid *Festulolium* cultivars investigated are derived from advanced breeding materials, *i.e.*, F_8 - F_9 generations (Kopecký *et al.* 2006).

The genome instability in the *Festulolium* allotetraploid hybrids listed above all refer to *Lolium* spp. in hybridisation to *F. pratensis*. It is possible that in less investigated and more distant *Lolium/Festuca* spp. hybrid combinations

that genome integrity may be greater.

Kopecký *et al.* (2018) reported that *L. multiflorum* × *F. arundinacea* var. *glaucescens* cv. Lueur (4x) has nearly equivalent amounts of chromatin from its parental species. Cv. Lueur, and more recently, *L. perenne* × *F. mairei* cv. AberRoot (4x) (M.W. Humphreys, unpublished results) are the only known amphiploid *Festulolium* cultivars to retain, to a large extent, a balanced genome constitution (*i.e.* at least over ca F_8 - F_9 generations). In addition to factors encouraging preferential chromosome pairing, incorporation of a functional chromosome-pairing regulator, proposed by Jauhar (1975) as an important stage in the evolution of fescue polyploids, would further improve hybrid genome integrity across generations, and support cultivar development.

Festulolium for ecosystem services

Grasslands are now increasingly recognised for their wide contributions to society and for their delivery of “public good” service, in addition to their better acknowledged roles for use in livestock agriculture (Abberton *et al.* 2008, Humphreys *et al.* 2014b). Amongst their numerous and diverse properties, grasslands provide carbon and water catchment provisions. They help regulate the acquisition and storage of rainfall in soils, and its subsequent release into river systems. They have an important role in preventing soil erosion and in assisting the regulation and retention of essential plant nutrients. Thereby, grasslands help to prevent river pollution and to maintain water quality. Grasslands are also important for *in situ* conservation of genetic resources, and are major providers of habitat for essential insect pollinators required for seed and fruit production by many crop species.

Festulolium cultivars have been developed to help maintain sustainable forage crop production, and through their resilience, to provide farmers some security against onsets of climatic stress. Although *Festulolium* cultivars have in the main involved only two fescue species (*F. pratensis* and *F. arundinacea*), they increasingly now derive from alternative species' combinations. Even when having the same species combination, cultivars may vary in the proportions of their constituent *Lolium* and *Festuca* genomes (Kopecký *et al.* 2006, 2018, Ghesquière *et al.* 2010a,b). Moreover, the parent species used to generate *Festulolium* cultivars are highly heterogeneous. Cultivars developed in different breeding programmes, even if the species' combinations are the same, will be genetically dissimilar. As a consequence, the extent of the various ecosystem services provided by *Festulolium* cultivars, will differ. If they are to achieve their intended function, it is essential that they are chosen and used only in the locations and for the purposes for which they were designed.

An important feature of perennial grasslands is the enormous biomass they produce below ground (Newman *et al.* 1989), a feature considered highly relevant to the provisions of ecosystem services (Bardgett *et al.* 2014). A survey of old grassland in the UK carried out by Dickinson and Polwart (1982) measured root biomass of around

5 t ha⁻¹ (0.5 kg m⁻²) in the top 15 cm of soil. Fescue species characteristically have larger and deeper root systems than ryegrasses (Coughan *et al.* 2017). *Festulolium* cultivars frequently demonstrate combined rooting characters found in their parent species. Like ryegrasses, *Festulolium* cultivars frequently have significantly higher root branching, in particular in soils near to the surface, than are found in fescues. However, compared to ryegrass, *Festulolium* generally have superior root biomass and root depth (Coughan *et al.* 2017, Humphreys *et al.* 2018). In a small but detailed study, Durand *et al.* (2007) using O¹⁸ demonstrated that *Festulolium* hybrids extracted water at soils depths inaccessible to *Lolium* cultivars, which would clearly assist them in providing greater drought tolerance.

Many ecological and environmental benefits emanate from the interactions between the roots of *Festulolium* and the soils in which they grow, for example, for flood mitigation (Macleod *et al.* 2013).

Until recently, grass breeding targets had focused only on the above-ground traits, mainly due to the difficulties in root phenotyping and selection. As a consequence, plant breeders have largely neglected improvements to root design and behaviour. New high-throughput root phenotyping technology is now becoming incorporated in plant selection, and into new breeding strategies. The incorporation of detailed root imaging undertaken at the National Plant Phenomics Centre (NPPC) in Aberystwyth is an example where detailed characteristics of root ontogeny and turn-over over entire growing seasons are now measured regularly, and with increasing efficiency, both in grasses and clovers (Marshall *et al.* (2016).

Festulolium cultivars have demonstrated their hidden values through their beneficial root-soil interactions. The first registered *Festulolium loliaceum* cultivar, cv Prior, produced in the 1970s has demonstrated a very timely ecosystem service, at a time of increased rainfall intensity. Through its deep rooting properties and significant root senescence at depth at the end of the growing season, cv Prior has demonstrated an ability to enhance soil structure and thereby increase soil porosity aiding soil water retention and mitigating surface flooding. This property was demonstrated within two consecutive multidiscipline BBSRC-funded programmes, initially using replicated field plots within the “SuperGrass” project (Macleod *et al.* 2013) project. Subsequently, the cultivar was tested both with or without white clover at the field scale, and including the use of livestock within the “SureRoot” project (BB/L009889/1; www.sureroot.uk).

Using hydrologically isolated field plots in the BBSRC “SuperGrass” project at Rothamsted Research North Wyke Research Station, it was demonstrated how cv. Prior, when compared to cultivars of its parental species, consistently reduced rates of surface run-off (a 70 % reduction compared to a registered perennial ryegrass cultivar, and a 43 % reduction compared to a meadow fescue cultivar). The results suggested a role for *Festulolium* for flood mitigation. The hypothesis was subsequently tested within the BBSRC “SureRoot” project. Using hydrologically isolated fields at the North Wyke Farm Platform (NWFP), an independent modelling study (Li *et al.* 2017)

demonstrated the benefits to soil hydrology from cv. Prior in delaying surface run-off, especially following extreme rainfall. The “SureRoot” project demonstrated that the benefits of *Festulolium* were even greater when grown in mixtures with white clover. Through root senescence, Li *et al.* (2017) also demonstrated that the *Festulolium* cultivar could contribute to carbon sequestration in soils. In their modelling study, *Festulolium* cv. Prior was predicted to have potential to fix more, and to lose less C through soil respiration than a current registered *L. perenne* cultivar. In their calculations, Li *et al.* (2017) suggested that soil C storage with reseeded cv. Prior gradually increases by 0.525 t(C) ha⁻¹ (0.052 5 kg(C) m⁻²) in two years following reseeded.

In order to enhance efficiencies in C soil deposition in grassland soils and for future benefit to the environment, C sequestration from grass root turn-over should focus primarily on those more biologically inert deep soil profiles (Kell 2011). Recent root research, such as that of Macleod *et al.* (2013) and Li *et al.* (2017), would suggest a role in this regard for deep rooting *Festulolium* cultivars.

Other research undertaken on working farms within the “SureRoot” project involved trials comparing field performance of the *Festulolium* cv. AberNiche, an Italian-ryegrass-like introgression cultivar and registered hybrid ryegrass cultivar as a control. Root biomass was found to be higher for cv. AberNiche when grown on farms with sandy soils. Similar to the outcomes reported in Macleod *et al.* (2013), the autumn water infiltration rates were higher across all farms and livestock systems in the *Festulolium* areas [30.7 ± 5.87(SEM) cm h⁻¹] compared to the ryegrass areas [16.6 ± 2.1(SEM) cm h⁻¹] (Powell *et al.* 2018).

Another outcome of the “SureRoot” project, (C. Marley, personal communication), was the finding that number of earthworms found was far greater in soils under *Festulolium* cv. AberNiche (*L. multiflorum* × *F. pratensis*) and cv. AberRoot (*L. perenne* × *F. mairei*) than in adjoining soils under ryegrass at the same farm location and under the same field managements.

The technologies now becoming available to researchers and breeders provide the means to monitor root growth throughout the growing season and offer new insight into grass crop development. Precision root-scan photography provided the first detailed comparisons in root ontogeny of ryegrass, *Festulolium* amphiploid hybrids, and *Festulolium* introgression lines throughout a growing season (Humphreys *et al.* 2018). An experimental amphiploid population of *L. perenne* × *F. arundinacea* var. *glaucescens* (Bx514), and of *Festulolium* cv. AberAtlas (*L. perenne* × *F. mairei*), both with forage growth equivalent to control *L. perenne* cultivar (Humphreys *et al.* 2014a), had significantly greater root biomass and root depth (Humphreys *et al.* 2018). The greater root growth of the *Festulolium* hybrids showed no negative impact to compromise their forage production.

Rootphenomics demonstrated that Bx509, a *Festulolium* population with a sequence of *F. arundinacea*-derived genes for drought tolerance in an otherwise complete genome of *L. perenne* cv. AberStar, had significantly greater root biomass than cv. AberStar, a likely explanation

for its superior drought tolerance (Humphreys *et al.* 2018).

In a *L. perenne* linkage-mapping population, Turner *et al.* (2008) demonstrated that QTLs associated with large root systems were also associated with drought tolerance and foliar growth. The findings, later supported by Humphreys *et al.* (2018), were that grasses with high forage production frequently have large root systems providing them some resilience to soil water deficits.

As far as the authors are aware, breeding to mitigate impacts from soil compaction derived either from livestock or machinery compressions and often exacerbated by prolonged droughts or floods, have until recently been absent. Soil compaction affects both soil and forage productivity. A recent investigation compared the impacts from machinery-derived soil compaction, both on field performance, and on underlying soil characters using plots with grass species of different root biomass, including three *Festulolium* cultivars (Muhandiram *et al.* 2020). Overall, findings showed that alternative grass root structures conveyed differing resilience to compaction. Amongst the *L. perenne*, *F. arundinacea* and *Festulolium* cultivars tested, it was a *L. multiflorum* × *F. arundinacea* var. *glaucescens* population (Bx514), that provided resilience to soil compaction without compromising forage productivity (Muhandiram *et al.* 2020).

However, the benefits of *Festulolium* are not restricted solely to the below ground properties, and, compared to ryegrass, include improved efficiencies in ruminant nutrition through better protein provision for livestock. An increase in the protein-use-efficiency by ruminants should result in reduced nitrogenous wastes and greenhouse gas emissions that are frequently associated with livestock agriculture (Humphreys *et al.* 2014, Kamau *et al.* 2020). There is growing evidence that *Festulolium* cultivars may provide some additional ecosystem services via their positive impacts on ruminant nutrition. Livestock agriculture is increasingly criticised for its negative impacts on the environment, and in particular, for the associated releases of nitrous wastes and greenhouse gases: ammonia, nitrous oxide and methane, a consequence of inefficient plant protein use by ruminants. This is largely due to plant-mediated proteolysis of fodder when ingested by ruminants and is catalysed by both microbial, but initially, endogenous plant proteases (Kamau *et al.* 2020).

Alternative grass breeding approaches have been undertaken over recent years to improve feed efficiencies in ruminants. The best known are the development of high sugar ryegrasses, designed to increase rumen microbes and through their activities, enhance the nitrogen uptake from fodder (Wilkins and Humphreys 2003). However, an alternative strategy that involves *Festulolium* instead of ryegrass, provides a possible complementary approach. This focuses on lowering of the initial protein intake by ruminants, and subsequently, to support ingested protein retention, in order to allow greater time for uptake by the animal and conversion into meat and dairy products. Through a series of *in vitro* studies, it has been demonstrated that certain fescue species, like *F. arundinacea* var. *glaucescens* have, compared to ryegrass, up to four times greater protein retention when subjected to rumen-like

conditions (Shaw 2006). The fescue trait for decreased rates of endogenous protein degradation remains expressed in certain amphiploid *Festulolium* cultivars. Moreover, this trait is accompanied by overall forage yields and quality equivalent to ryegrass (Humphreys *et al.* 2014a, O'Donovan 2015).

Under simulated rumen-conditions, both the amphiploid *Festulolium* population Bx514 (*L. multiflorum* × *F. arundinacea* var. *glaucescens*, and *Festulolium* cv. AberRoot (*L. perenne* × *F. mairei*) demonstrated significantly lower plant-mediated proteolysis than their ryegrass control (Kamau *et al.* 2020). Although no differences in microbial protein synthesis were observed during early fermentation (0–6h after feeding), an increase in microbial N flow in cv. AberRoot (+30%) and in Bx514 (+41%) was observed during late fermentation (6–24h after feeding). There were higher overall microbial N flows (+13.5% for cv. AberRoot, and +20.2% for Bx514) when compared to the ryegrass control (Kamau *et al.* 2020). These observations indicate the potential for *Festulolium* cultivars that express the slow plant-mediated proteolysis trait to improve on efficiencies of forage protein capture and to decrease the releases of N pollutants onto the land.

The research described above focused only on freshly harvested *Festulolium* plant material and its impacts on ruminant nutrition. Very recently, this has been extended further on whether similar benefits might be evident in 90 day-old silage (Muhandiram *et al.* 2020). The initial findings are promising and indicated that crude protein content obtained from silage generated from cut foliage of the *Festulolium* population Bx514 (*L. perenne* × *Festuca arundinacea* var. *glaucescens*) was significantly higher than corresponding silage obtained from a high yielding current high sugar ryegrass cultivar.

Conclusions

Grasslands are multifunctional; using a holistic breeding approach, new cultivars would be evaluated not only as fodder for livestock, but also for the provision and range of important ecosystem benefits. It is increasingly necessary to develop high yielding cultivars with resilience against the various climate-change scenarios they may encounter. *Festulolium* cultivars may provide the necessary phenotype plasticity that brings such multiple benefits and bespoke options that can be tailored to the specific requirements of farmers and society. By incorporating new high-throughput phenomic and genomic technologies, complex advantageous traits may be assembled together in single cultivars.

The geographic range of *Festulolium* could expand in future years due to their resilience to abiotic stresses and to their ecosystem services. They offer potential sustainable high yielding and high-quality forage grasses that can bring economic and social benefits to farmers in developing countries. For example, a recent study undertaken by farmers in Kenya compared the field performance of IBERS-bred grasses; two UK National Listed perennial ryegrass cultivars and five *Festulolium* cultivars (Mwendia

et al. 2019). The grasses were compared over one growing season on clay loam soils at Ol-joro-Orok in the central highlands of Kenya at about 2600 - 2800 m a.s.l. An evaluation by 61 local dairy farmers rated the grasses on criteria they nominated as being important, including dry matter yield, growth rate, height, frost tolerance, disease tolerance and leafiness. The *Festulolium* cultivars collectively outperformed the ryegrass cultivars. Mwendia *et al.* (2019) concluded that the grasses had definite potential for future commercial use in grassland livestock systems in Kenya. The results in Kenya, and elsewhere show there is an increasing range of new opportunities for use of *Festulolium* cultivars.

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